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Two new experimental techniques focus on the kinetics and mechanism of ion permeation through channels in bilayer membranes. Laser doppler velocimetry, optimized for the study of ion flows in membrane channels, generates the velocity distribution for these ions. This velocity distribution provides detailed kinetic information on the mechanism of ion permeation.

Modulation of the absorption and fluorescence spectra of Tl(I) ions in membrane channels using a pulse sequence or sinusoidal transmembrane potential permits the detection of small changes in intrachannel Tl(I) as a function of membrane potential. The technique is used to determine the fraction of total current which is carried by Tl(I) ion when the bathing solution contains mixtures of K(I) and Tl(I) ions. These data are used to elucidate the mechanisms of permeation and channel blockage by these ions.

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RESEARCH OBJECTIVE

The observation of ion motions in membrane channels and its attendant shot noise are electrically limited by the capacitative charging time of the membrane. Since such data are essential for a thorough understanding of the ion permeation mechanism and kinetics, an alternative photophysical technique is used to provide direct observations of the ion motions within the channel. Laser doppler scattering, in which the laser light frequency is doppler-shifted by the moving ions, constitutes a non-invasive technique for observing ion motions in the channels. The average transit velocity and the spread of observed velocities about this average translate to a detailed molecular picture of the ion permeation process.

Since Tl(I) ion has different absorption and fluorescence properties in aqueous solution and gramicidin channels, differential techniques are used to observe the total Tl(I) ion in gramicidin channels. These data, in conjunction with the net channel current, permit determination of the fraction of the current carried by Tl(I) ion. These data can elucidate the nature of the Tl(I) permeation mechanism.

BACKGROUND

Laser doppler velocimetry has been used as a non-invasive technique to study gas and liquid motions. Such motions are often observed by seeding the fluid with homogeneous particles which are large effective scatterers. The technique has also been applied to biophysical applications including blood flow and protoplasm streaming.

The laser doppler frequency shift for these large scattering centers is generally obtained by splitting a laser beam into two equally displaced, equal amplitude beams and refocusing them on the small volume containing the moving particles. The intersecting beams form an interference pattern. A scattering particle, moving laterally through this pattern, produces a scattering intensity modulated by the velocity at which the particle traverses the regions of high and low intensity in the scattering volume. The system can easily detect velocities from mm/sec to m/sec and generally uses autocorrelation detection when the density of scattering particles is small.

Observation of the motion of large ions like Tl(I) in gramicidin channels requires novel new instrumentation. Although each Tl(I) ion constitutes a small scattering center, a large number of such ions move simultaneously through an ensemble of membrane channels in a membrane in the laser interference volume. Because the laser wavelength is long compared to the channel length, the resultant scattering simultaneously records all velocities for ions within the channels and no ion synchronization is required.

The instrumental system developed to detect the scattering from the permeant ions was based on a modulation scheme used by Gudeman, Begeman, Pfaff and Saykally, Phys. Rev. Letters 50,727 (1983)) to detect small concentrations of specific ions in a plasma. These workers applied a sinusoidal electric field to a plasma tube to modulate velocities only of

charged species. The modulation of the absorption signal was then detected using a lock-in amplifier. The charged species could be detected with minimal background contribution from the neutral species.

Similar modulation techniques are also applied to detect small changes in absorbance and fluorescence when $Tl(I)$ ions traverse a gramicidin channel. Since the ion population in the channel will change as the transmembrane potential changes, the detection system can be synchronized with a temporal applied potential to detect very small absorbance and fluorescence changes.

FIRST YEAR PROGRESS

The frequency of scattered radiation from a moving object will be doppler-shifted. This signal can be mixed with a reference beam or scattering from a second angle to give a difference frequency proportional to the particle velocity. This difference frequency is generated when the two beams are mixed on the surface of a photomultiplier. Because the scattering signal from all the moving ions will be small, the instrumentation selected for detection uses lock-in detection tied to a time dependent transmembrane potential. The sinusoidal transmembrane potential produces both an amplitude modulation and a frequency modulation of the difference frequency. Since the population of ions in the channel can vary with the transmembrane potential (an assumption which will be verified with the absorbance modulation instrumentation), the amplitude of difference frequency will follow the amplitude of the transmembrane potential. The difference frequency is detected with a tuned amplifier which can be scanned through a range of difference frequencies. This difference frequency is amplitude demodulated to give a low frequency signal proportional to the population of ions generating the difference frequency. The amplitude of this signal is detected with the lock-in amplifier.

The change in velocity with changing transmembrane potential frequency modulates the difference frequency at the transmembrane potential frequency. This signal can also be demodulated for a range of difference frequencies and now gives a signal proportional to the variation in velocity at each of the transmembrane potentials. Although this frequency modulation detection scheme should have greater noise immunity, detection is complicated by the amplitude modulation background and the amplitude modulation detection scheme is preferred.

The shape of the observed distribution provides a direct measure of the variations of velocity within the channel. For example, a perfectly homogeneous channel permits ions to flow at a single velocity for a given transmembrane potential. This will translate to a narrow velocity distribution.

During this study, two possible interpretations for the difference frequency have become apparent. The predicted transit times for single ions in a channel require a shot noise frequency in the 20 Mhz range. Velocity calculations based on this time and the length of the channel predict difference frequencies in the range of 0.5 Mhz. Both these

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frequency domains can be detected with the tuned amplifier and are being studied while more detailed models for scattering from an ensemble of moving ions are developed.

Because of its general utility, a dual beam input system was selected. The laser beam is separated into two beams which are displaced 25 mm from the optical axis of the laser beam in the horizontal plane. The two beams are then focused to the region containing the membrane channel. In the simplest interpretation, the crossed beams form an interference pattern. An ion moving through this pattern will modulate the scattering intensity at a frequency proportional to the ion velocity through the interference pattern. Although each individual ion represents a small scattering center, the synchronous motion of a large number of such ions approximates the motion of a large scattering center.

A monolein membrane, oriented 45° to the optic axis, separates two equal volume bathing solutions. Quartz plates permit the entrance the reference beams and the exit of the scattering beam.

A second "modulation" technique permits differentiation of K(I) and Tl(I) ions in gramicidin channels when these ions flow under an applied potential difference. The resultant electrical current gives the total ion flux but not the portion of that flux carried by each ion. These specific ion fluxes require photophysical observation of Tl(I) in the channels. The aqueous Tl(I) ion absorbs ultraviolet radiation from 200 - 250 nm. However, the absorption is shifted approximately 20 nm to the red in less polar solvents. Absorption spectra of methanol solutions containing 10^{-5} M concentrations of Tl(I) and gramicidin show a significant increase in absorption at 270 nm compared to methanol solutions of either gramicidin or Tl(I). A similar increase in absorption is observed for a shoulder at 200 nm. However, since the 270 nm matches a high intensity emission peak of a xenon-mercury arc lamp, this wavelength region is selected.

The absorption at 270 nm is modulated by applying a time-dependent potential difference across the membrane. Although the change in absorption as Tl(I) ions enter the channels could be detected using a lock-in amplifier locked to a sinusoidal transmembrane potential, a two pulse system was selected because of an available signal averager. The bathing solutions contain 1 M KAc and 1 M mixtures of KAc and TlAc respectively. A two pulse sequence is used to (1) move K(I) ions into the channels (2) allow Tl(I) ion from the mixture to enter the channels. A change in absorption during the first pulse indicates the displacement of Tl(I) ions from the channels. The absorption change for the second pulse is proportional to the number of Tl(I) ions in the channels for a given transmembrane potential.

The modulation technique is being used with bathing mixtures of Tl(I) and K(I) to clarify several anomalous experimental observations. For example, although Tl(I) is an excellent permeant ion at high mole fraction, it tends to block channel currents at low mole fractions (Neher, Biophys. Biochem. Acta 401,540 (1975)). The experimental determination of fractions of current carried by each ion should clarify the mechanism which produces these effects. In addition, the actual permeation mechanism for Tl(I) may differ from that for the alkali cations (Andersen and Procopio, Acta

Physiol. Scand. Suppl. 481,27 (1980)). Because of the strong change in absorption, the system was redesigned from a fluorescence system to an absorption system for this summer.

FUTURE PROSPECTS

The major thrust of this project is the development of instrumentation which will permit observation of ion fluxes and ion populations in channels. The photophysical methods required by this proposal are new and may involve some modification for optimal results. The laser doppler technique has not been used to study the coherent motion of a large number of smaller scattering centers. The membrane channels, however, constitute the best system for such a study. The channels restrict the "slower" observable ion motions to the membrane region so these motions can be selectively observed in isolation from other ion motions. This isolation is further enhanced by the special amplitude and frequency modulation techniques. The doppler-shifted frequency becomes the "carrier" frequency but it is modulated at a lower frequency to permit detection with lock-in amplification.

In addition to the crossed beam technique, we are considering several reference beam techniques in a search for the optimal scattering signal. The alternate techniques utilize the fact that the membrane molecules act as a stationary scattering reference which can then be mixed with the scattering from the ions moving relative to this membrane. These alternate configurations will be explored fully.

Since membrane electrostriction may contribute to the observed Doppler scattering, bilayer membranes without gramicidin will be studied. Such studies will also establish if the Tl(I) can permeate the membrane as TlCl. Cl^- ion is present at low concentration for the Ag-AgCl electrodes.

The system will also be tested by observing the motion of small numbers of larger molecules in the membrane. Scattering by the tetraphenylborate anion will be probed using only the lock-in amplifier for this slower diffusion process. The system provides a model system to check the optical arrangement of the system.

The laser doppler experiments will give a distribution of velocities for ions within the channels. These data must be associated with a theoretical map of ion motions within the channel. Does the ion slow significantly at a "binding site" within the channel? Is a model which postulates two such binding sites near the membrane interfaces a valid description of the ion permeation process? Such questions require a detailed study of ion distributions within the channel. A paper which establishes such distributions in multisite channels will be submitted shortly. Future work will involve models which provide a direct correlation between theoretical models of ion permeation and the experimentally observed velocity distribution.

The modulated absorption system provides a novel technique for determination of fractions of ions which carry currents in the gramicidin channel. The experiments will establish the mechanism which permits low

concentrations of Tl(I) to limit transmembrane current while permitting high concentrations of Tl(I) to function as a non-saturable permeant species. While the detection system has been modified for absorption measurements, similar experiments can be performed using the Tl(I) fluorescence. In such cases, the Tl(I) emission varies due to changing absorption as the transmembrane potential is changed. Since the total light reaching the photomultiplier is much smaller in this case, the technique may prove more sensitive.

The experiments will be extended to mixtures of Tl(I) and other alkali ions to establish a more complete picture of the permeation mechanism. In all cases, the focus will rest on a study of ions in the channels, not the channels themselves.